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# Applying soft matter techniques to solve challenges in cryopreservation

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Cryopreservation allows the long-term storage of biological material, and has become integral for numerous applications including assisted reproductive technologies, stem cell therapies, blood banking, and species preservation. However, there are still hundreds of cell types that cannot be cryopreserved, and no organs at all. The biggest challenge facing cryopreservation is the ongoing reliance on predominantly just two cryoprotectants (dimethylsulfoxide and glycerol) as well as physical limitations to heating and cooling rates. The field of soft matter can accelerate cryopreservation research by providing insights into the underlying mechanisms and interactions of cells, cryoprotectants, and solvents including the role of temperature. With techniques as far ranging as differential scanning calorimetry, atomic force microscopy, and neutron and X-ray scattering, it is possible to probe multiple length and time scales in order to identify and characterise important interactions. This in turn can lead to the development of new cryoprotectants, and new methods of heating/cooling which could overcome some of the current challenges of cryopreservation.

## KEYWORDS

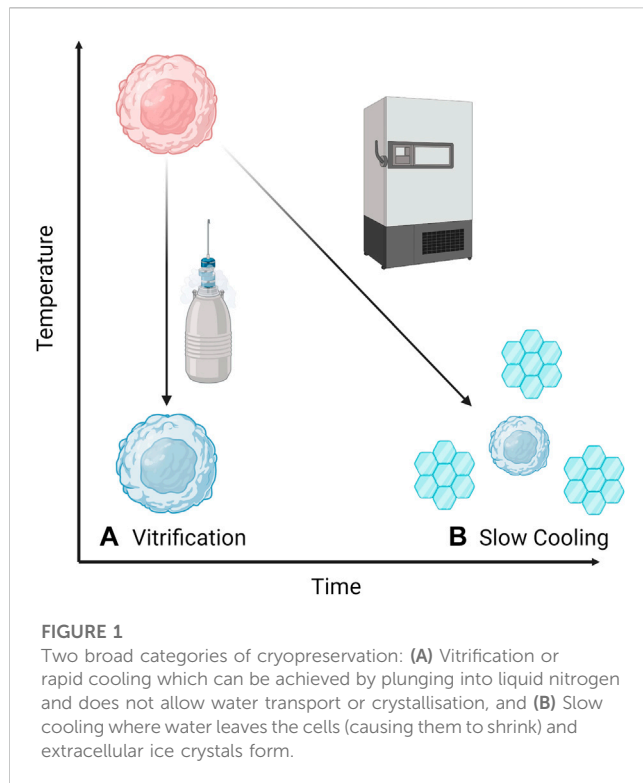
cryopreservation, soft matter, cryoprotectant, characterization techniques, chemical interactions

## 1 Introduction

### 1.1 What is cryopreservation?

Cryopreservation is the process of cooling biological samples down to very low temperatures, usually below  $-80^{\circ}\text{C}$ , so that they can be stored for a long time (Mazur, 1970; Mazur, 2004). At such low temperatures, biological processes cease to operate, thus allowing the sample to enter a kind of ‘stasis’ where it theoretically remains unchanged until it is warmed (Mazur, 1970, 2004). Generally, there are two broad categories of cryopreservation (Figure 1); slow cooling, whereby the rate of cooling is low enough that extracellular ice forms and some water is transported out of the cells; and vitrification whereby the rate of cooling is so fast that water transport cannot occur and there is no ice formation but rather the entire system solidifies in a non-crystalline state (Mazur, 1970; Pegg, 2007).

In nature there are numerous organisms that have adapted to extreme conditions, including extreme cold, so that they are able to enter a natural kind of “stasis” state. Almost universally, this adaptation includes the production of a cryoprotectant (CPA) that protects the organism’s cells from damage during freezing (Storey, 1990; Fuller et al., 2004). In nature, the primary cryoprotectants are small sugars, in particular the disaccharides trehalose and



sucrose. Trehalose conveys both cold and desiccation resistance in many species, including bacteria and tardigrades, while sucrose is predominantly associated with plant freezing tolerance (Koster and Lynch, 1992; Koster et al., 1994). The primary modes of action are the ability to vitrify, combined with their ability to protect membranes at low to moderate dehydrations (Koster et al., 1994; Wolfe and Bryant, 1999; Koster et al., 2000). The mechanisms for membrane protection have been debated, with the resolution (Andersen et al., 2011) appearing to lie in the level of hydration. At the low to moderate hydrations most relevant to cryopreservation, sugars act osmotically to retain water and keep membranes separated during dehydration (Koster and Bryant, 2006; Lenne et al., 2006; Lenne et al., 2007; Lenne et al., 2009). At the very low hydrations mostly relevant for desiccation and freeze-drying, sugars replace water, thus stabilising proteins and lipids (Coutinho et al., 1988; Crowe et al., 1990; Crowe et al., 1992; Kandror et al., 2002; Crowe et al., 2004; Nguyen et al., 2022).

Antifreeze proteins and glycoproteins have been found to play a role in numerous cold and freeze-resistant species, particularly fish and insects (Balcerzak et al., 2014). These solutes act to protect organisms from freezing damage through thermal hysteresis and inhibiting ice recrystallisation (Balcerzak et al., 2014). The effect of this can be significant, for example, the freezing point of blood from arctic fishes is routinely 1°C–2°C below zero (Scholander et al., 1957; DeVries et al., 1970; DeVries, 1971). Similarly, a xylomannan glycolipid has been found in a diverse range of cold-tolerant taxa including amphibians, insects and plants and is thought to bind to lipid membranes to prevent damage during freezing (Walters et al., 2011). Other species accumulate different chemicals to protect them from freezing, such as wood frogs which produce urea,

glycerol and glucose which allows them to tolerate temperatures as low as –16°C (Costanzo et al., 2013; Larson et al., 2014; Costanzo et al., 2015). In addition to animals, some plants have also evolved freeze tolerance, either through dehydration strategies or production of intracellular CPAs, including antifreeze proteins (distinct from those described above) (Hon et al., 1994; Pearce, 2004; Preston and Sandve, 2013).

In nature these molecules are produced inside cells and therefore can act intracellularly. However, the molecules are generally not membrane permeable, and therefore when used for artificial cryopreservation can only act extracellularly. Thus alternative membrane permeating cryoprotectants are needed for most laboratory cryopreservation. Note that cryopreservation for some types of cells and biomolecules can be achieved with non-penetrating CPAs (called extracellular CPAs), discussed briefly below.

Laboratory cryopreservation of microbial and fungal species has been carried out for close to a century (Haines and Barcroft, 1938; Fulton and Smith, 1953; Fennell, 1960), however, this review will focus on cryopreservation of plant and animal cells. The first report of artificial cryopreservation of an animal cell occurred early in the 20th century, although pinning down the very first reference is difficult (Shettles, 1939; Shaffner, 1942). These first studies focused on spermatozoa, which showed excellent recovery when frozen with glycerol—the first significant CPA (Polge et al., 1949). Since then there have been several breakthroughs in cryopreservation research, including the discovery of dimethylsulfoxide (DMSO) as a CPA, which is now the most widely-used for mammalian cells (Lovell and Bishop, 1959). Research over the last century has made cryopreservation a standard laboratory practice, which varies somewhat between cell types, but broadly involves adding a CPA to the cells, then cooling the mixture at a specified rate followed by low-temperature storage, usually at either –80°C or –196°C (Pegg, 2007). When required, cells are thawed by warming (some variation for different cell types) and usually washed to remove the CPA which would otherwise have toxic effects on the cells (Sputtek and Sputtek, 2004; Pegg, 2007). Cryopreservation along these lines is important in numerous fields and for a wide variety of applications.

### 1.1.1 Applications

Cryopreservation has applications in numerous fields including assisted reproductive technology, species preservation, and biomedicine (Mazur, 1970, 2004; Pegg, 2007; Jang et al., 2017; Xie et al., 2022). Some of these applications are well-developed, while others are just emerging. The applications of cryopreservation are limited only by the types of cells that one wants to store.

One of the first applications of cryopreservation for animal cells was for assisted reproduction (Polge et al., 1949; Hungerford et al., 2023). This technology has come a long way and it is now possible to store sperm, oocytes and even embryos from a range of species (Polge et al., 1949; Bosch et al., 2020; Daly et al., 2020; Ferré et al., 2020; Stoecklein et al., 2021; Hungerford et al., 2023). However, the cryopreservation of reproductive tissue from some species, in particular amphibians, reptiles and marsupials, has remained challenging (Clulow and Clulow, 2016; Rodger, 2019; Campbell et al., 2020).

TABLE 1 Common CPAs, their uses, and toxicity.

Intracellular CPA	Example uses	Apparent toxicity	References
Dimethylsulfoxide (DMSO)	Gold standard for many mammalian cells, including stem cells, lymphocytes and research cell lines (e.g., HeLa and CHO) Bovine red blood cells	Epigenetic changes, Adverse events in people following transfusion, Changes to cell membrane	Lovelock and Bishop. (1959); Benekli et al. (2000); Zenhäusern et al. (2000); Iwatani et al. (2006); Ataseven et al. (2017); Tunçer et al. (2018); Awan et al. (2020); Lysak et al. (2021); Raju et al. (2021)
Glycerol (Gly)	Mammalian (including human) spermatozoa, Human red blood cells	Oxidative stress, more toxic than other CPAs for certain cell types	Polge et al. (1949); Smith, (1950); Chen and Tian. (2005); Scott et al. (2005); Korrapati et al. (2012)
Ethylene Glycol (EG)	Mammalian embryos (including human and bovine)	Less toxic than most other intracellular CPAs, Upregulation of stress-related genes	Pollock et al. (1991); Mukaida et al. (1998); Sommerfeld and Niemann. (1999); Cordeiro et al. (2015)
Propylene Glycol (1,2-propanediol) (PG)	Endothelial cells, Limbal stem cells	Relatively low toxicity—approved for food and drug use, Inhibited development of mouse embryos	Damien et al. (1990); von Bomhard et al. (2016); Osei-Bempong et al. (2018)
Methanol (Meth)	Fish sperm, Chilled storage of fish embryos,	Neurotoxic, Mitochondrial dysfunction	Rico et al. (2006); Zampolla et al. (2009); Desai et al. (2015); Bozkurt et al. (2019)
Butanediol (1,3- and 2,3-butanediol) <sup>a</sup>	Red blood cells (human)	Almost 25% reduction in cell survival of vascular endothelial cells after 9 min exposure, significantly better than DMSO which caused more than 60% reduction, Minimal toxicity to red blood cells	Mehl and Boutron. (1988); Boutron, (1992); Wusteman et al. (2002)
(BD)			
Proline-Glycerol (deep eutectic solvent) (Prol-Gly)	Mammalian cells (THP1, HaCat, UG87-MG, PC3)	Caused 50% cell death after ~6 h exposure at 10 wt%	Bryant et al. (2022)
Extracellular CPA	Example uses	Apparent toxicity	References
Sucrose (Suc)	Sheep erythrocytes, CHO	Not reported	Mazur et al. (1969); Hoffmann and Brogden, (1976)
Polyvinylpyrrolidone (PVP)	CHO, Human peripheral lymphocytes, Rat pancreatic islets Adipose-derived stem cells	Toxic to stem cells above 20% Completely lethal to mouse embryos	Mazur et al. (1969); Ashwood-Smith et al. (1972); Damjanovic and Thomas, (1974); Kuleshova et al. (2001); El-Shewy et al. (2004); Thirumala et al. (2010)
Hydroxyethyl Starch (HES)	CHO Red blood cells (including human) Stem cells <sup>b</sup>	Dependent on molecular weight and molar substitution, Minimum toxicity recorded	Ashwood-Smith et al. (1972); Sputtek et al. (1994); Stolzing et al. (2012)
Trehalose (Tre)	3T3 Murine fibroblasts, <sup>c</sup> Human keratinocytes, <sup>c</sup> Platelets	Not reported	Eroglu et al. (2000); Wolkers et al. (2001)

<sup>a</sup>There are differences in toxicities and efficacies between isomers.

<sup>b</sup>As an additive with DMSO or other intracellular CPA.

<sup>c</sup>Required addition of a membrane pore-forming toxin in order to facilitate intracellular uptake.

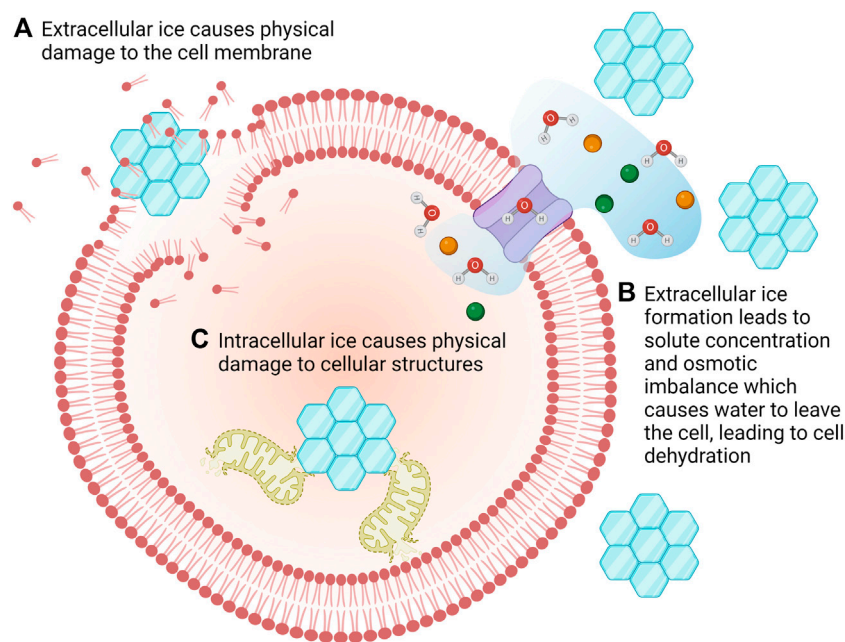
For application in species preservation, samples of reproductive tissues, embryos, or seeds are taken from live specimens and cryopreserved. This allows for long-term storage of specimens as a “back-up” to their living (and perhaps endangered) counterparts (Walters et al., 2004; Clulow and Clulow, 2016). One of the most outstanding examples of this is the Svalbard Global Seed Vault which contains samples of more than 5,000 species from around the world (Asdal and Guarino, 2018). This facility could allow the re-establishment of plant species in the event of mass extinction.

In addition to the above applications, the discovery and development of cryopreservation techniques has allowed unprecedented advances in science. Cryopreservation enables the storage and long-term access of biological specimens, including immortalised cell-lines (Geraghty et al., 2014). These cell lines are used globally for drug testing, disease investigations, and as

therapeutic agents (e.g., stem cells) which in turn has led to unprecedented advances in all of these scientific fields.

### 1.1.2 Current state of cryopreservation

Currently, cryopreservation can be used to store a relatively limited number of cell types. The majority of existing cryopreservation protocols rely on a small group of established CPAs, generally require small sample volumes, and there is very little scope for optimisation of cooling and warming protocols. All of these limitations contribute to the current inability to store hundreds of cell types, including some neural cells and cardiomyocytes (Jang et al., 2017), primary natural killer (NK) and T cells (Yao and Matosevic, 2021), as well as an inability to store any complex tissues or organs (Jacobsen et al., 1984; Bakhach, 2009; Lewis et al., 2016). These limitations are outlined below.



**FIGURE 2**

There are multiple mechanisms of freezing damage. The formation of extracellular ice crystals can cause mechanical damage to cells (A) but it also results in concentration of solutes which causes water to flow out of the cells in an attempt to restore osmotic balance. This can lead to desiccation/dehydration of the cells (B). Intracellular ice crystals damage intracellular organelles leading to cell death (C).

The majority of current cryopreservation protocols rely on variables that are not optimised for specific cell types, including CPA and cooling rate. Part of this is due to the complex, and resource-intensive nature of developing new cryopreservation protocols which relies on ready access to significant quantities of good-quality cells, and appropriate facilities.

One of the most difficult challenges to overcome in cryopreservation is the reliance on a limited number of CPAs (see Table 1), the most common being dimethylsulfoxide (DMSO) and glycerol (Best, 2015; Elliott et al., 2017; Raju et al., 2021). Recent evidence suggests that DMSO is more toxic than previously thought (Zenhäusern et al., 2000) and could in fact be causing epigenetic changes to cell lines which may have implications for subsequent applications (Iwatani et al., 2006; Tunçer et al., 2018). Similarly, glycerol leads to oxidative stress (Korrapati et al., 2012) and has been shown to be more toxic than other CPAs for certain cell types such as flounder embryos (Chen and Tian, 2005). Furthermore, a reliance on toxic CPAs such as DMSO is one of the main hurdles preventing storage of complex tissues and organs. As described above, for successful cryopreservation cells must be treated with a CPA prior to freezing. This is not an issue in single-cell suspensions where all cells are exposed to the CPA simultaneously. However, with complex tissues and organs there is a diffusion limitation where extended lengths of time are required for the CPA to penetrate to deeper cell layers (Martinez-Madrid et al., 2004; Pegg, 2007), and in the mean-time the outer layers are damaged by CPA toxicity. Thus, one of the biggest challenges for extending cryopreservation from single-to multi-cell systems is delivery of CPA to inner cell layers without toxic effects to the outer cell layers (Pegg, 2007; Bleisinger et al., 2020).

The second biggest challenge in the field of cryopreservation stems from technological limitations to cooling and heating rates.

Theoretically, if a sample could be instantaneously cooled to  $-196^{\circ}\text{C}$  and instantaneously warmed to room temperature, there would be little to no risk of freezing damage as there would be no time for ice to form (Gao and Critser, 2000; Mazur, 2004). While the cooling side of cryopreservation has been improved with the invention of controlled-rate freezers and the ability to plunge samples directly into liquid nitrogen or other cryogens (Wolfe and Bryant, 2001; Bojic et al., 2021), thawing generally still relies on convective warming by placing a sample into a  $37^{\circ}\text{C}$  water bath. This process is sufficiently slow that it puts the sample at risk of recrystallisation damage. Furthermore, it is impractical for larger sample vials, or tissues and organs which are multilayered (Manuchehrabadi et al., 2017; Bojic et al., 2021).

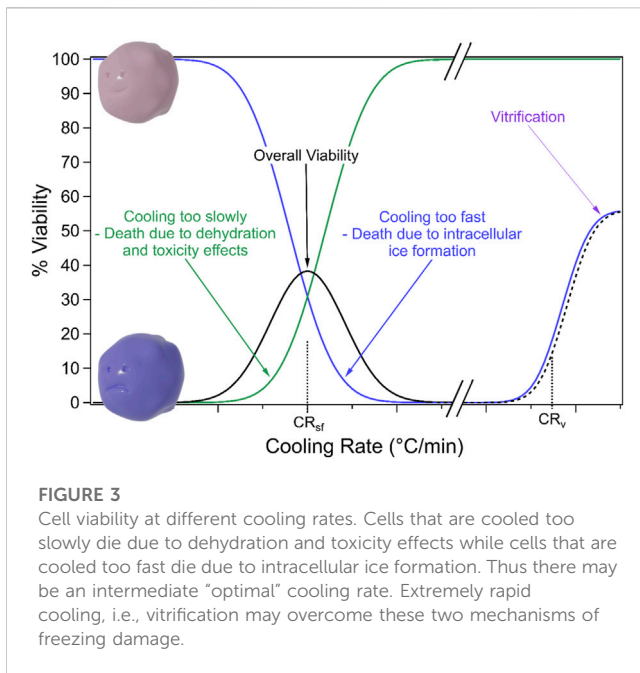
Until the issues of CPA toxicity and cooling/warming rates are addressed, extending cryopreservation beyond the cell types that can already be stored will be slow. However, if these, and other, challenges can be overcome, then it is possible that hundreds of new cell types can be stored, and perhaps even tissues and organs. Recent research into the major challenges of cryopreservation is discussed below.

## 2 Cryopreservation challenges

### 2.1 Freezing damage

#### 2.1.1 What is freezing damage?

Low temperatures are not in themselves damaging to most cell types. Rather, it is the physical process of freezing which is damaging (Mazur, 1963; Mazur, 1977; Wolfe and Bryant, 2001). This is due to



**FIGURE 3**

Cell viability at different cooling rates. Cells that are cooled too slowly die due to dehydration and toxicity effects while cells that are cooled too fast die due to intracellular ice formation. Thus there may be an intermediate “optimal” cooling rate. Extremely rapid cooling, i.e., vitrification may overcome these two mechanisms of freezing damage.

a combination of mechanical damage due to the formation of intra- and extra-cellular ice crystals, and solute damage due to changes in the concentration of electrolytes (Figure 2) (Wolfe and Bryant, 2001).

At sub-zero temperatures water will form ice. However, this is a stochastic process which means that the probability of ice formation is proportional to the volume of water available; ice is more likely to form in a large volume than a small volume (Bryant, 1995). Thus, when considering cryopreservation of a cell, ice is more likely to form in the extracellular environment than the intracellular (Karow JR., 1969). However, intracellular ice formation is somewhat dependent on cooling rate; at slow cooling rates if ice forms externally, water will be transported out of the cell thus increasing the intracellular solute concentration resulting in dehydration but not freezing of the intracellular space. In contrast, if a cell is cooled too quickly, it will not lose sufficient water, resulting in supercooling and eventual formation of intracellular ice (Mazur, 1977).

The preferential formation of ice in the extracellular environment leads to an increase in solute concentration (salts, sugars, proteins, etc.) within the unfrozen fraction (Lovell, 1953a; b). This increased concentration can be damaging to cells for a number of reasons, including creating an osmotic imbalance leading to osmotic shock, changing protein conformation, or influencing the bilayer structure of the cell membrane (Mazur, 1970; Steponkus, 1984; Gao and Critser, 2000; Wolfe and Bryant, 2001; Mazur, 2004; Elliott et al., 2017; Zhao and Fu, 2017). Furthermore, as the extracellular unfrozen fraction becomes more concentrated, the osmotic imbalance forces water out of the cell, leading to dehydration which can lead to membrane phase changes and cell damage (Crowe et al., 1990; Crowe et al., 1992; Bryant, 1995; Wolfe and Bryant, 1999; Wolfe and Bryant, 2001; Wolfe et al., 2002).

While extracellular ice formation is generally harmless to dilute cell suspensions, it can become lethal when cells are closely packed

as in concentrated suspensions or multi-cellular assemblies (Pegg, 2020). Previous work has demonstrated that extracellular ice causes structural disruption of tissues including muscle (Elford and Walter, 1972; Taylor and Pegg, 1983; Jalal and Zidi, 2018), cartilage disks (Rendal-Vázquez et al., 2001; Pegg et al., 2006) and kidneys (Jacobsen et al., 1984). The location and degree of ice formation is related to the rate of cooling and warming, providing potential mechanisms for controlling ice formation (Fahy, 1990; Pegg, 2020).

### 2.1.2 Cooling/warming rates and freezing damage

The contrasting mechanisms of freeze damage (solute effects vs. ice damage) led Mazur to develop the “Two Factor Hypothesis” (Mazur, 1963; Mazur et al., 1972). This hypothesis relates cooling rates to cell survival, suggesting that there may be an ideal intermediate cooling rate that maximises cell survival. This hypothesis is illustrated in Figure 3. At low cooling rates cells die due to dehydration and toxicity from high solute concentrations. However, at high cooling rates, intracellular ice formation may occur which will also be lethal. Thus, an intermediate cooling rate should lead to maximum cell survival. The value of the ideal cooling rate varies between cell types due to factors such as size and permeability (Mazur, 1963; Mazur, 1977).

Freezing damage may also be combatted through vitrification. Vitrification is a transition to a solid glass-like state without ice formation and requires very rapid cooling, as shown in Figure 3 (Gao and Critser, 2000; Bojic et al., 2021). In general, vitrification depends on high concentrations of CPA to reduce the cooling/warming rates required to prevent ice formation, which then raises toxicity issues (Bojic et al., 2021).

It should be noted that ice formation and associated freezing damage can happen during warming (often called recrystallisation) as well as cooling. Therefore warming rate is an important consideration in combatting freezing damage (Gao and Critser, 2000; Waters et al., 2020). In particular, a sample that has undergone vitrification may be especially susceptible to ice recrystallization if the warming rate is too slow (Bojic et al., 2021; Zhan et al., 2022).

#### 2.1.2.1 Heat transfer limitations

One of the biggest limitations to heating and cooling rates is heat transfer. Ideally, samples for preservation would be cooled and warmed instantaneously, thus achieving vitrification and re-warming without ice formation. In practice, cooling rates are limited by the physics of heat transfer.

Currently, vitrification relies on directly plunging samples into liquid nitrogen to achieve cooling rates of  $\sim 340^\circ\text{C}/\text{min}$  for small organs like ovaries (Courbiere et al., 2006) and  $10,000^\circ\text{C}/\text{min}$  for single cell suspensions (Nawroth et al., 2002). However, even with this method there is an uneven temperature change for the outside of the sample compared to the inside which for large samples (such as organs) can lead to fracturing and cell death (Steif et al., 2007).

Most cryopreservation protocols rely on slow warming in a hot-water bath which for 1 mL of cells in a 2 mL cryovial results in a warming rate of  $\sim 45 \pm 8^\circ\text{C}/\text{min}$  (Baboo et al., 2019) (significantly slower than the cooling rates used in vitrification). This method of warming has two significant limitations: 1) the slow warming rate can lead to ice recrystallisation, especially for samples that have been vitrified (Sharma et al., 2021) and 2) there is an uneven temperature

change across samples (outside will be warmer than inside), which becomes more pronounced with larger samples (Manuchehrabadi et al., 2017). Uneven temperatures lead to cracking and fracturing during both cooling and warming (Steif et al., 2007; Sharma et al., 2021). Thus, a protocol which is suitable for a 1 mL solution of cells may result in very low cell recovery if applied to a 5 mL sample.

To overcome the above limitations of convective warming, substantial research is being carried out into new methods of sample warming which can achieve faster warming rates and can simultaneously warm an entire sample, regardless of size. For liquid samples such as blood, this can be achieved by maximising surface area (Sputtek and Mingers, 1999; Sputtek et al., 2007). Other methods include:

- Nanowarming which utilises iron oxide nanoparticles excited by a radio frequency field (Etheridge et al., 2014). This technique has been used to warm vitrified rat kidneys at a rate of 63.7°C/min (Sharma et al., 2021) and resulted in a higher viability of cryopreserved porcine arteries compared to those warmed using traditional convective methods (Manuchehrabadi et al., 2017). Importantly, the warming rates of tissues can be substantially different to the warming rate of the media on its own (Manuchehrabadi et al., 2017).
- Infrared radiation which can achieve various warming rates depending on power and length of exposure (Cao et al., 2022; Zhan et al., 2022). Adherent cells have been successfully thawed using this method with warming rates of 3,000°C/min (Bissoyi and Braslavsky, 2021).
- Laser warming which can achieve phenomenal heating rates up to 10 million °C/min. This technique has been utilised to warm mouse oocytes (Jin et al., 2014) and zebrafish embryos (Khosla et al., 2020).
- Using a water bath with additional microwave warming, which increased the rate from 70.2°C/min to 105°C/min while reducing the temperature gradient (Ruan et al., 2020).

## 2.2 Cryoprotectants

As discussed above, CPAs are usually added to media prior to freezing. These CPAs inhibit ice formation, promote vitrification, and can act as buffers against solute toxicity induced by dehydration (Meryman, 1971; Hubálek, 2003; Fuller, 2004; Best, 2015; Elliott et al., 2017). Table 1 shows a summary of the most commonly used CPAs, along with some novel CPAs that have recently been being explored. The table includes notes on the cell types the CPAs are used for, and their toxicity. CPAs generally fall into two categories; intracellular or permeating and extracellular or non-permeating (Karow JR., 1969; Fuller, 2004; Elliott et al., 2017; Raju et al., 2021). Extracellular cryoprotectants include sucrose, polyvinylpyrrolidone and hydroxyethyl starch. These molecules are unable to enter cells and instead increase the osmolarity of the extracellular environment which causes cell dehydration and reduces the formation of intracellular ice crystals (Meryman, 1971; Raju et al., 2021).

DMSO and glycerol are two of the most commonly used intracellular CPAs, and both have been known for more than 50 years (Polge et al., 1949; Lovelock and Bishop, 1959).

However, despite decades of research, there are still hundreds of cell types that cannot be efficiently preserved using these CPAs, including granulocytes (Sputtek and Sputtek, 2004) and T cells (Yao and Matosevic, 2021). In addition only very simple tissue architectures such as blood vessels have been preserved to date, and these have generally had poor recovery post-cryopreservation (Bakhach, 2009).

### 2.2.1 Toxicity

One significant limitation to cryopreservation of cells, and in particular tissues and organs is CPA toxicity (Table 1) (Fahy, 1986; Raju et al., 2021). Toxicity of CPAs leads to cell death during incubation and cryopreservation and requires rapid and time-consuming washing steps (Shu et al., 2014). For example, the IC50 (concentration which results in a 50% inhibition in growth) of DMSO for epithelial cells was found to be just 2.9% (Layman, 1987), and near 2% for human leukemic cells (Hajighasemi and Tajic, 2017) which is significantly less than the concentrations commonly used for cryopreservation (5%–10%). In fact DMSO has been linked to epigenetic changes in cultured cells which may impact on downstream applications such as embryo development (Iwatani et al., 2006; Tunçer et al., 2018). In addition, CPA toxicity can impact medical treatments, including adverse reactions. For example, fatal cardiac arrhythmia (Zenhäusern et al., 2000); neurotoxicity (Windrum and Morris, 2003; Ataseven et al., 2017); and respiratory depression (Benekli et al., 2000) have been recorded in patients administered cells stored with DMSO (Shu et al., 2014).

CPAs must, by necessity, interact with water and cellular components in order to facilitate protection during cryopreservation. However, new CPAs with reduced toxicity could overcome many of the existing challenges (Raju et al., 2021). For example, a CPA with little to no toxicity could be perfused through an organ or tissue and allowed time to fully penetrate all cell layers before freezing. This is not an option with current toxic CPAs as outer cell layers which are exposed for an extended period of time will die.

### 2.2.2 Permeability

In addition to toxicity, permeability is another challenge for CPAs. As discussed above, naturally occurring CPAs are produced in the cells of freeze-resistant organisms, so do not need to permeate to perform their function. So while these natural compounds are sometimes used as non-permeating CPAs, an intracellular CPA must also usually be included (Dou et al., 2019; Sui et al., 2019).

Thus, one key requirement of effective CPAs is that they are able to permeate into cells. Small, moderately polar molecules can generally enter cells via passive diffusion such as DMSO and glycerol (Yang and Hinner, 2015). Larger, or more polar or ionic species cannot passively diffuse, which is why sugars such as trehalose are non-permeating CPAs (Yang and Hinner, 2015). The likelihood of a molecule being permeable can be roughly estimated using the “rule of 5” which states that poor permeability is likely if: there are more than 5 hydrogen bond donors; there are more than 10 hydrogen bond acceptors; the molecular weight is greater than 500 g/mol; and the lipophilicity (CLogP) is >5 (Lipinski et al., 1997; Yang and Hinner, 2015).

**TABLE 2** Techniques that are standard for cryopreservation research and will not be discussed further in this review.

Technique	Brief description	Example results	References
Differential Scanning Calorimetry (DSC)	Measures temperature and heat flow of a sample compared to a reference	Thermal transitions (e.g., freezing and melting points and glass transitions) with different heating/cooling rates. Critical cooling/warming rates	Stacy et al. (2019), Courbiere et al. (2006) Mehl, (1993) Bryant et al. (2021); Bryant et al. (2022); Pakhomov et al. (2022)
Molecular Dynamics	Atomistic computer simulations	Locations and interaction of cryoprotectants with cellular components (membranes, proteins, etc.)	Bryant et al. (2022) Malajczuk et al. (2022a) Malajczuk et al. (2022b) Rhys et al. (2022)
Electron Microscopy	High resolution imaging	Changes to cells on exposure to cryoprotective agents or the cryopreservation process. Location, size, and growth patterns of ice crystals	Gosden et al. (2010); Keskin et al. (2020)

Some molecules can enter cells via transporter-mediated entry including ions, water, some sugars, and most amino acids (Yang and Hinner, 2015). When developing CPAs they must be able to get into the target cell through either passive diffusion or transporter-mediated entry.

### 2.2.2.1 Improved permeation of existing CPAs

One avenue of research is to improve the permeability of existing CPAs. Trehalose has been a particular focus for this strategy. Numerous methods have been applied to trehalose to improve permeation; including chemical modification (e.g., addition of an alkyl chain) (Abazari et al., 2015), nanoparticle delivery (Rao et al., 2015; Stefanic et al., 2017), pseudopeptides (Chen et al., 2020), microinjection (Eroglu et al., 2002) and genetic modification (Guo et al., 2000).

An alternative method is to alter the cell membrane to allow trehalose permeability. This can be achieved by a simple increase in temperature in the case of platelets (Wolkers et al., 2001), through addition of permeability-increasing biopolymers (Lynch et al., 2010), electroporation (Dovgan et al., 2017), or through the application of bacterially-derived pore-forming molecules (Eroglu et al., 2000). In the latter case, an increased survival of mammalian cells was observed with low trehalose concentrations following cryopreservation, but higher concentrations resulted in massive cell death (Eroglu et al., 2000).

Evidently, there are many potential strategies to increase CPA uptake, however, each of these come with disadvantages such as the potential toxicity or downstream effects of altering the cells (either genetically or by introducing pores), difficulties in scaling (e.g., for microinjection), and concentration and toxicity concerns for nanoparticles. Thus a more fruitful avenue of research may be to find new, permeating CPAs.

### 2.2.3 Search for new CPAs

As demonstrated from the discussion above, there is a vital need for new, permeating, less-toxic CPAs, not only to overcome the issues associated with current CPAs, but also to store new cell types, and perhaps eventually more complex assemblies such as tissues and organs. The ideal CPA will inhibit ice formation and promote vitrification, permeate into cells, and prevent protein/membrane damage, without being itself too toxic. A detailed discussion of the need for rational design of new CPAs is given in our recent review (Raju et al., 2021).

There are several classes of chemicals that have shown promise as CPAs, although more research is required to explore their ability to store new cell types (Raju et al., 2021). These potential new CPAs

include anti-freeze proteins (Leclère et al., 2011; Balcerzak et al., 2014; Kuiper et al., 2015), glycolipids (Bendas et al., 1996; Walters et al., 2011), carbohydrate derivatives (Eroglu et al., 2000; Chaytor et al., 2011; Imamura et al., 2014; Abazari et al., 2015; Raju et al., 2019b), ionic liquids (Bryant et al., 2021), deep eutectic solvents (Castro et al., 2018; Jesus et al., 2021; Bryant et al., 2022) and others (Sasaki et al., 2005; Toyosawa et al., 2009; Miyamoto et al., 2012; Verdanova et al., 2014).

Testing the cryoprotective efficacy of new CPAs can be costly and time-consuming. Therefore, rational methods of design and testing must be employed, as described in our recent review (Raju et al., 2021). Soft matter techniques in particular can be used to screen potential CPAs for promising properties before carrying out full-scale testing. These properties include permeability, thermal behaviour, and interactions with cells or model cellular components, as discussed below.

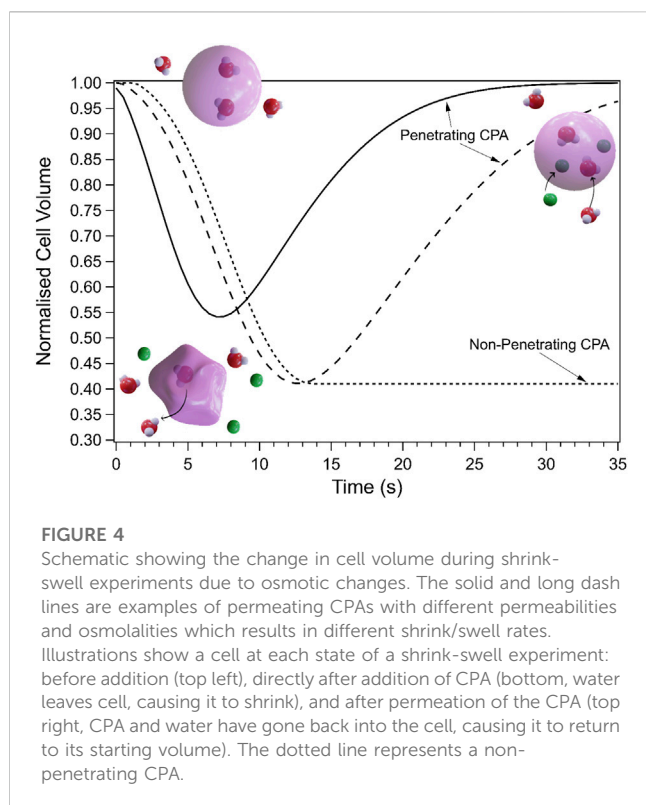
## 3 Soft matter techniques for improved cryopreservation

Soft matter encompasses the study of materials that lie somewhere between a liquid and a solid, and by definition includes most biological systems including cells and tissues (Tang, 2022). The multi-disciplinary nature of this field makes it ideally suited to the exploration of cryopreservation because it allows the investigation of the biological, chemical, and physical processes that are at work. Numerous techniques are capable of interrogating soft matter systems, all with different capabilities, accessible length scales and limitations, as outlined below.

There are a broad range of techniques which are standard in the cryopreservation literature as outlined in Table 2. These include optical and electron microscopy techniques, differential scanning calorimetry and so on. This review will focus on techniques which are less widely used, and/or have had significant recent developments which make them exciting techniques for cryobiological investigations.

### 3.1 Shrink-swell experiments

Permeability is a critical requirement for intracellular CPAs, as discussed above, and can be measured in several ways as reviewed by McGrath (McGrath, 1997). These methods have advantages and disadvantages that depend on cell type, availability, and the conditions being examined. However, one of the simplest



methods of measuring CPA permeability is the “shrink-swell” light microscopy experiment. In this technique, changes to cell size on exposure to solutions containing CPAs are observed using microscopy. Addition of a CPA solution causes an osmotic imbalance that forces water out of cells and causes them to shrink. If the CPA is permeable, it will subsequently flow into the cells, reversing the osmotic imbalance and causing the cell to swell again. However, if the CPA cannot permeate, then the cell will remain in its shrunken state (Mazur and Schneider, 1986; Kleinhans, 1998; Bryant et al., 2022). This data is quantified by measuring the cell area over time (e.g., using software such as ImageJ to measure cell area in different frames), converting that to a volume, and then normalising to the starting volume of the cell (Bryant et al., 2021; Bryant et al., 2022), as shown in Figure 4. Most studies rely on time-consuming manual area determinations and data processing, however recent advances in computational image analysis and machine learning provides avenues for automated image analysis which will significantly speed up the process and allow upscaling of the technique (Shu et al., 2016; Tu et al., 2022). A limitation of this method is that it assumes the cells are spherical for conversion of area to volume, which is often not the case. This assumption may lead to erroneous conclusions about shrink-swell behaviour.

The shrink-swell experiment described above relies on the cells being fixed in place for the duration of the experiment (e.g., by a poly-L-lysine coating). This can be difficult and unreliable, but also may introduce new behaviours that would not occur in unfixed cells. Thus, this experiment can be improved by using microfluidic technologies (Figure 5) (Zhao and Fu, 2017; Raju et al., 2019a). Perfusion chambers (Chen et al., 2007) and single-cell traps (Tan and Takeuchi, 2007; Wlodkowic et al., 2009) have been developed to capture cells and allow permeability measurements without fixation (Chen et al., 2007; Kitson

et al., 2012; Edashige, 2017; Fang et al., 2017; Tseng et al., 2022; Tu et al., 2022).

Other methods for determining shrink-swell behaviour include:

- Coulter electronic conductance which measures electrical conductivity between two chambers (Higgins and Karlsson, 2008a; Higgins and Karlsson, 2008b, 2010). The presence of a cell passing through an aperture causes an increase in resistance and results in a measured voltage spike which can be related to the cell size (Hirsch and Gallian, 1968; Model, 2018). The benefit of this method is that it is largely independent of cell shape, unlike the light microscopy method described above which generally assumes spherical cells. However, as the Coulter method relies on conductivity, the results can be complicated by the addition of solvents (as when testing permeability of potential CPAs) as the conductivity of the solvent itself will change.
- Flow cytometry utilises light scattering from particles in a flowing solution and has been applied to monitor cell volume changes (Noiles et al., 1997; Yeung et al., 2002; Tzur et al., 2011; Model, 2018). The technique allows rapid analysis of whole populations, unlike light microscopy which only analyses a handful of cells at a time. However, volume changes to cells can also cause changes to the refractive index, which changes scattering intensity and therefore can interfere with the apparent cell volume.

While cell volume can be measured in a variety of other ways, such as atomic force microscopy (discussed below) and confocal microscopy, these techniques are not appropriate for permeability measurements as the imaging time is too slow. In the case of highly permeable CPAs such as DMSO, the entire shrink-swell process may be over in just 30 s, and therefore only techniques which allow rapid data acquisition are appropriate.

Data from shrink-swell experiments can be modelled to extrapolate the permeability parameter of potential CPAs. Models of this behaviour have progressed from one parameter (solute permeability) (Mazur et al., 1974; Mazur and Miller, 1976), to two (water and solute permeability) (Jacobs, 1933), to three (water and solute permeability, interaction parameter) (Kedem and Katchalsky, 1958). The three-parameter or Kedem-Katchalsky (KK) formalism is most commonly used today as it accounts for the interaction between solute and solvent (Kleinhans, 1998). The resulting permeability parameters can then be used to rationally tailor cryopreservation protocols. First it can be used to exclude molecules which are non-permeating from further consideration, and second it will yield permeability parameters for those that are. If permeation is rapid (as is often the case for DMSO) then the cells will require little to no time to uptake the CPA prior to freezing. However, if the permeability is low, then permeation into the cell will be slow, and therefore the cell may require a period of “incubation” with the CPA prior to freezing in order to allow sufficient uptake to have a protective effect (Bryant et al., 2022).

### 3.2 Langmuir isotherms

Langmuir isotherm experiments utilise a simple Langmuir trough (Figure 6) to compress monolayers of amphiphilic



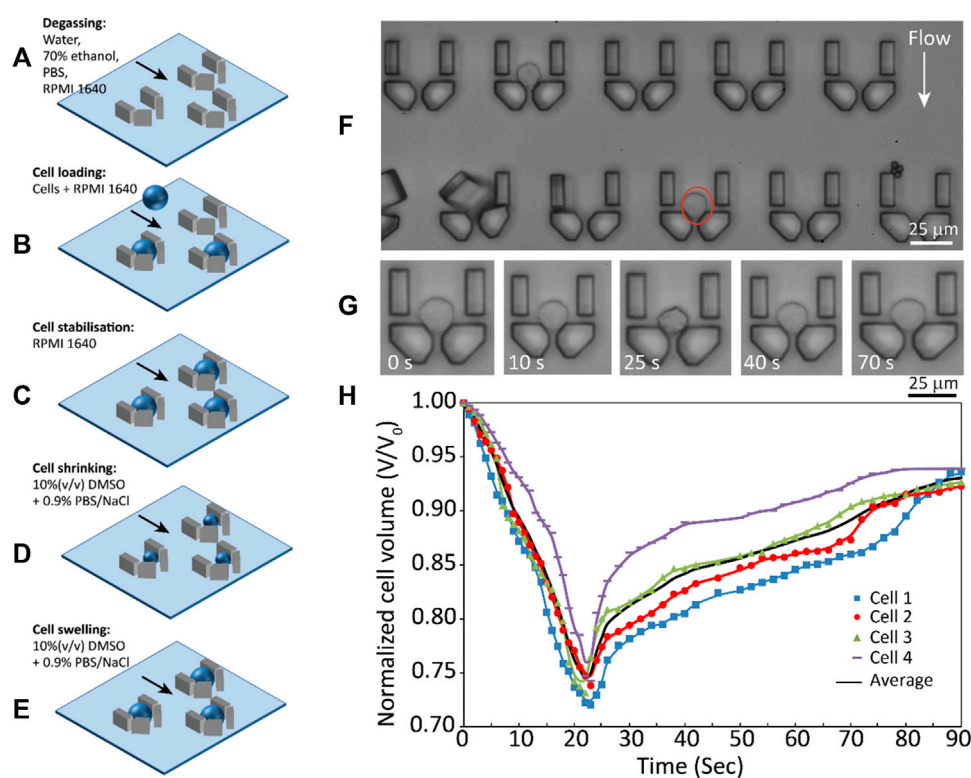


FIGURE 5

Example of a microfluidic device used to trap cells for subsequent shrink-swell analysis. (A) degassing and channel filling, (B) cell loading, (C) cell stabilization, (D) cell shrinking after DMSO addition, (E) cell swelling as DMSO penetrates, (F) cell traps, (G) time-lapse images showing volume changes of a THP-1 cell, (H) volume kinetics of four THP-1 cells with average shown in black. Reprinted from (Raju et al., 2019a), with the permission of AIP Publishing.

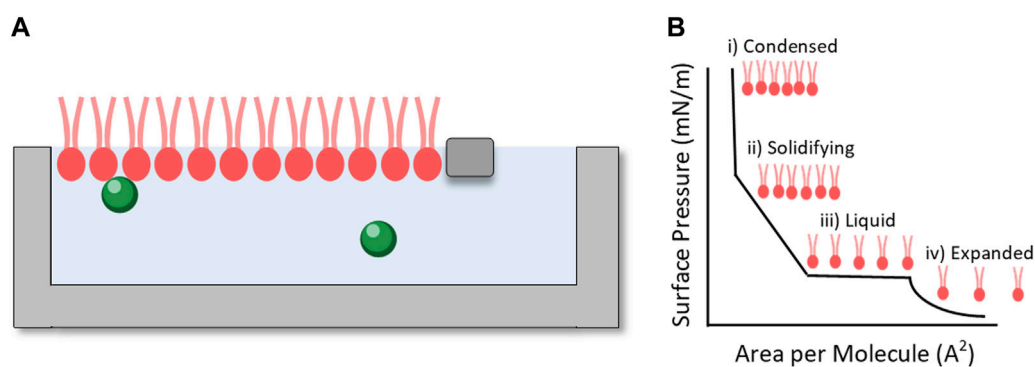
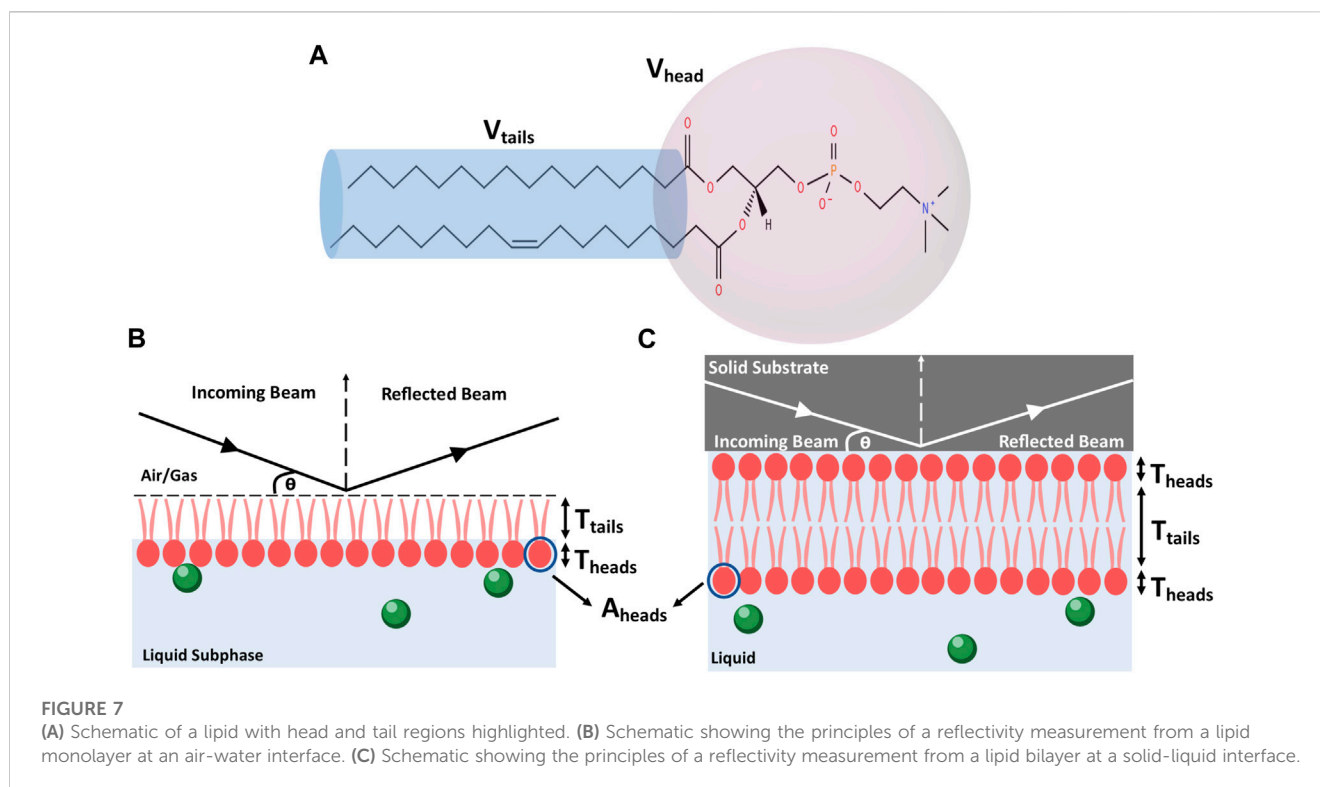


FIGURE 6

(A) Langmuir trough setup illustrating a lipid monolayer (pink) between two moveable barriers with chemicals of interest dissolved in the subphase (green spheres). (B) Illustrative pressure isotherm showing changing area per molecule with changing surface pressure.

molecules on the surface of a subphase. The apparatus usually measures surface pressure using a Wilhelmy plate and is equipped with barriers which can be moved closer together or further apart to control the surface pressure and area. These experiments are frequently used to examine lipid behaviour under different conditions such as temperature and lateral pressure (Oliveira et al., 2022). The lipid of interest is dissolved in a volatile

solvent such as ethanol or chloroform and then injected onto the surface of the subphase, usually water (within the “barriers” as shown in Figure 6). The volatile solvent evaporates and the lipid assembles at the air-solvent interface so that the hydrophobic tails are orientated into the air (Oliveira et al., 2022). The barriers can then be moved to measure changes in surface pressure with compression to ensure that a stable monolayer has formed at



the surface. At this point changes can be made to the system (e.g., temperature, or the injection of an additive into the subphase) and the response of the monolayer is measured as a change in surface pressure. These values can then be used to calculate the area per molecule of lipid as a function of different conditions.

Langmuir isotherms have revealed that sugars with higher complexity have a more significant impact on lipid monolayers than simpler ones (e.g., trehalose compared to glucose) by increasing the apparent headgroup area (Clark et al., 2015). Conversely, the polymer hydroxyethyl starch has no interaction with lipids, consistent with its role as an external non-permeating CPA (Bryant et al., 1994).

Another study found that the effect of permeating CPAs on model lipid monolayers was dependent on the lipid species, with a more significant impact observed for unsaturated compared to saturated lipids (Raju et al., 2020). The same study observed a significant increase in headgroup area for all lipids with addition of glycerol and ethylene glycol, but only for saturated DPPC with addition of DMSO. This finding has implications for other studies that have focused on only one type of lipid as the results may not apply to cell membranes which are composed of many different types of lipids. In contrast to the above, a study utilising octadecanol as the lipid found that DMSO and glycerol did not significantly affect the pressure isotherms, and suggested that the cryoprotective mechanisms came from interactions either with membrane proteins or via interaction with the aqueous environment surrounding the lipids (Williams and Harris, 1977).

Langmuir isotherm experiments offer a simple benchtop approach to investigating the interaction of CPAs with model monolayers. It is limited by relying on a lipid monolayer at the solvent-air interface, rather than a bilayer which may more

accurately reflect the cell membrane. The technique is further complicated as the surface pressure can influence the degree of interaction observed between CPAs and the lipid layer, which may not be relevant to real membranes (Dabkowska et al., 2014). Despite this, these experiments can still shed light on interactions with lipid headgroups, such as whether molecules insert into the monolayer or cause an effective dehydration (and thus decrease in apparent headgroup area).

In addition to the uses described above, a Langmuir trough combined with Langmuir-Blodgett deposition can be used to prepare bilayer samples for a range of experiments including neutron and X-ray techniques to extract structural information about how CPAs may affect bilayer structure.

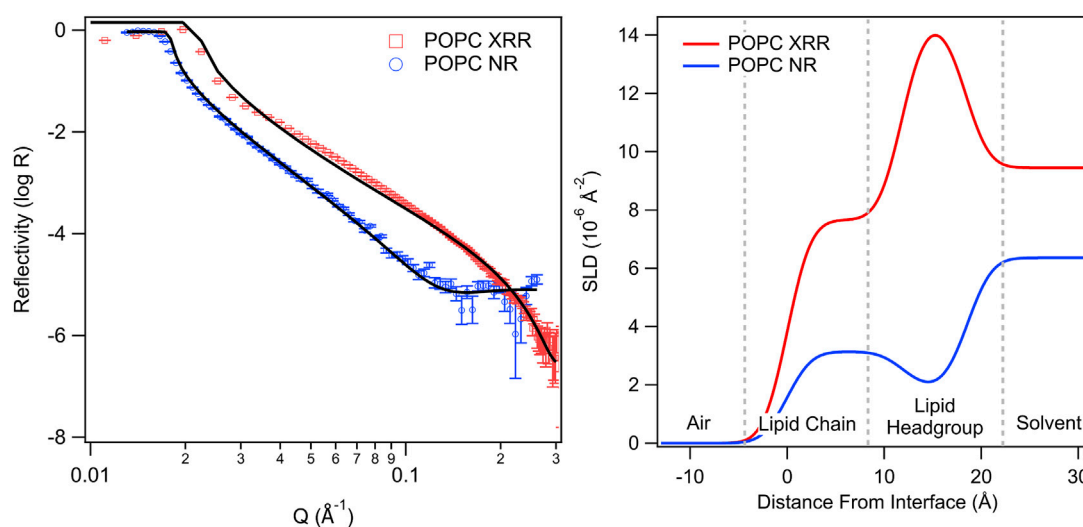
### 3.3 Neutron and X-ray scattering techniques

#### 3.3.1 Reflectivity

Reflectivity involves measuring the reflections off a surface. This reflectivity is measured as a function of the scattering vector  $q$  which can be defined as:

$$q = \left(\frac{4\pi}{\lambda}\right) \sin \theta$$

where  $\lambda$  is the wavelength of the incident neutrons or x-rays and  $\theta$  is the angle of incidence relative to the film plane (Krueger, 2001). Reflectivity provides information on the composition and thickness of layers at an interface under different conditions e.g., as a function of temperature or in the presence of additives (Daillant et al., 2005). Figure 7 shows an example of a lipid bilayer system that could be examined using reflectivity to determine interactions with CPAs.



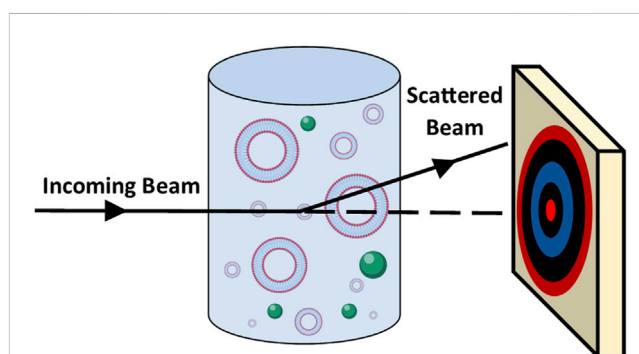
**FIGURE 8**

X-ray (XRR) and neutron (NR) reflectivity profiles (left) and associated SLD profiles (right) for POPC lipid monolayers with an aqueous subphase (adapted from Bryant et al., 2023, licensed CC-BY-4.0). The SLD profiles highlight differences in contrast between X-rays and neutrons, which can be used to localise different regions. Deuteration of molecules and/or the aqueous layer provides an additional contrast profile for neutrons.

Neutron and X-ray reflectivity rely on differences in the scattering length density (SLD) or “scattering power” of components within the sample. The greater the difference in SLD between components (e.g., a lipid head group and the solvent), the greater the resolution will be from these techniques. For neutrons, scattering arises from the nuclear scattering lengths while for x-rays scattering arises from electron density. Thus, in x-ray reflectivity experiments  $\text{H}_2\text{O}$  and  $\text{D}_2\text{O}$  are indistinguishable, but they are very different in neutron reflectivity experiments (Lakey et al., 2022). Thus, the two techniques can be used in tandem to “see” different parts of a sample. This provides independent measurements of the same parameters (e.g., layer thickness) and allows simultaneous fitting (Vaknin et al., 1991). In this case, known variables are fixed (e.g., SLDs) while the unknown variables are allowed to vary (e.g., layer thickness) but the resulting fit must match both the neutron and X-ray reflectivity data, providing greater precision and reliability in the results. An example is shown in Figure 8.

In neutron reflectivity a technique known as “contrast matching” can also be used to achieve multiple independent measurements of the same variable to improve analysis and fitting results. This technique relies on the significant difference in SLDs between hydrogen and deuterium, providing a stark contrast between  $\text{H}_2\text{O}$  ( $\text{SLD} = -5.6 \times 10^{-7} \text{ \AA}^{-2}$ ) and  $\text{D}_2\text{O}$  ( $\text{SLD} = 6.3 \times 10^{-6} \text{ \AA}^{-2}$ ). By adjusting the  $\text{H}_2\text{O}/\text{D}_2\text{O}$  ratio, the solvent SLD can be adjusted to the desired value, for example, to ‘match out’ the scattering from a component (e.g., the lipid hydrocarbon tail). Thus, the same measurement can be performed using solvents with different SLDs to get several independent measurements from the same system but highlighting different features. Then these multiple patterns can be simultaneously fit (fixing known variables such as the solvent SLD) to obtain a reliable value for the unknown parameters (Bayerl et al., 1990).

Reflectivity techniques have been used to examine lipid monolayers and bilayers under a variety of conditions. Such experiments have yielded insight into the hydration and positioning of the glycerol



**FIGURE 9**

Schematic showing the principles of small angle scattering whereby an incoming beam passes through the sample and is scattered onto a two-dimensional detector.

backbone of lipids in the fluid compared to condensed state (Bayerl et al., 1990) and the positioning of biomolecules such as proteins within a model lipid bilayer (Fragneto et al., 2000; Hossain et al., 2017; Hsieh et al., 2018). This gives insight into behaviours such as preferential partitioning into the headgroup (or tail) region, and broad changes to the lipid layer (e.g., change to thickness).

A handful of papers have used reflectivity to examine the interactions of CPAs with lipids. In one study a lipid monolayer of dipalmitoylphosphatidylcholine (DPPC) exposed to DMSO (0.1 and 0.05 mol fraction) showed no change in tail or headgroup thickness according to neutron reflectivity (Dabkowska et al., 2014). A different study used several techniques, including neutron reflectivity, to explore the interaction of sugars (glucose, glycerol and trehalose) with a DPPC monolayer. They found it difficult to fit their reflectivity data, but found it suggestive of glucose dehydrating the headgroup region (Clark et al., 2015).

Reflectivity offers an underutilised opportunity to better understand the interactions of CPAs with cell membranes. Based on the studies performed to date, it is likely that a bilayer system will yield better resolution and more relevant results than monolayers. However, future studies could utilise reflectivity to explore how potential CPAs interact with a wide range of lipids, including lipid mixtures to better represent real membranes, thus shedding light on interactions of CPAs and laying the groundwork for rational design of next-generation CPAs.

### 3.3.2 SAXS, WAXS, and SANS

Small and wide angle X-ray scattering (SAXS and WAXS) and small angle neutron scattering (SANS) are carried out in transmission with the scattered beam measured on a 2-dimensional detector (Figure 9). The sample's structural information is represented in reciprocal space by the elastic scattering pattern (Liu et al., 2022). After radial averaging, the scattering intensity can be given as a function of the scattering vector,  $q$ :

$$q = \left(\frac{4\pi}{\lambda}\right) \sin\left(\frac{\theta}{2}\right)$$

Where  $\theta$  is the scattering angle relative to the transmitted beam, and  $\lambda$  is the wavelength. As with reflectivity, the scattering is determined by the SLDs of the components in the sample. The scattering pattern is also influenced by the “structure factor” which is the interaction between scatterers in non-dilute systems.

SAXS is a widely used technique for studying structure in self-assembling systems, in particular the structure of lipid membranes (Nagle and Tristram-Nagle, 2000; Tyler et al., 2015). However, to date there have been relatively few studies using SAXS to understand cryobiological processes. One study using both SAXS and SANS showed that DMSO acts to dehydrate lipid headgroups (Kiselev et al., 1999; Shashkov et al., 1999). Studies involving one of the authors used SAXS/WAXS to show that the presence of vitrified sugars lowers the lipid gel to fluid transition temperature by tens of degrees (Koster et al., 1994), quantified the effects of different sugar concentrations on the transition temperature (Lenne et al., 2007), and produced a quantitative model to explain the effect (Lenne et al., 2009). A later study used SAXS to understand the interactions between lipid membranes and intrinsically disordered proteins implicated in cold tolerance in plants (Thalhammer et al., 2014).

Several studies have used SANS to explore the interaction of DMSO with lipids, usually in the form of multi- or uni-lamellar vesicles. One study used contrast variation SANS to show that DMSO can induce membrane phase transitions. DMSO also caused a decrease in interbilayer spacing of multilamellar vesicles (Gorshkova and Gordeliy, 2007). Two studies by the same group demonstrated that DMSO interacts strongly with water, causing dehydration of the intermembrane space and a subsequent decrease in repeat spacing (Kiselev et al., 1999; Shashkov et al., 1999).

Studies involving one of the authors have used contrast variation SANS to estimate the location of sugars in lipid/water mixtures (Lenne et al., 2006), and showed that glucose is excluded from the inverse hexagonal phase, which occurs during dehydration in some lipid systems. The utility of SAXS and SANS as tools to study the interactions of cryomolecules with membranes was reviewed in (Garvey et al., 2013).

### 3.3.3 Membrane diffraction

Membrane diffraction measures X-ray or neutron diffraction from self-assembled stacks of hundreds of bilayers (Wiener and White, 1991; Hauss et al., 2005). In this technique, by varying the deuteration of water, lipid and solute, the specific location of solutes within membranes can be determined to high precision, providing insights into the detailed interactions between the solutes and the membranes. In the context of cryobiology, this has been used to show that the natural cryoprotectants trehalose and sucrose do not preferentially interact with membrane headgroups at moderate hydrations (Kent et al., 2014; Kent et al., 2015), and has been used to measure how deeply cold regulated proteins penetrate into the lipid bilayer (Bremer et al., 2017).

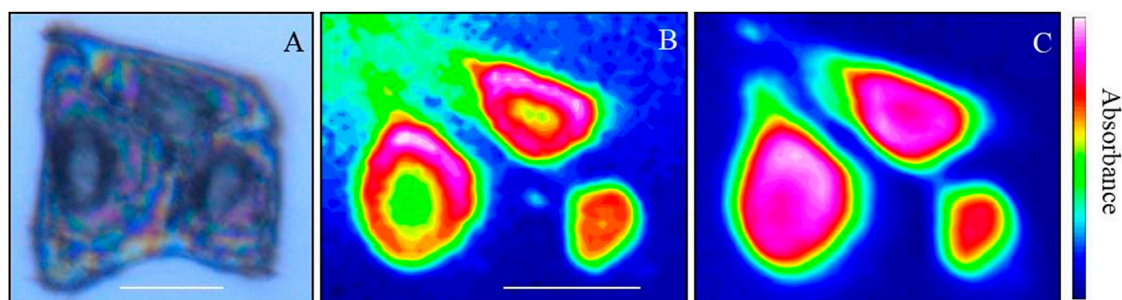
One of the main limitations of neutron (and high precision X-ray) scattering is the requirement for a specialist facility, usually with limited access. For neutrons in particular, fluxes tend to be low, requiring relatively long measurement times (~ tens of minutes to hours). Furthermore, contrast variation measurements require deuterated components which can be expensive, or completely unavailable, thus limiting the systems that can be explored. Despite these limitations, these techniques are critical tools and show a lot of promise for overcoming challenges in cryopreservation—in particular to understand the interactions of CPAs with model lipid bilayers.

## 3.4 FTIR spectroscopy and microscopy

Infrared (IR) and Near-IR spectroscopy measures the absorption, emission, or reflection of IR radiation as it interacts with matter in the region of ~4,000 to ~600  $\text{cm}^{-1}$  (Capuano and van Ruth, 2016; Pasquini, 2018). It can be used to interrogate the chemistry of solid (Chabal, 1988), liquid (Arrondo et al., 1993), or gas samples (Sharpe et al., 2004; MacAleese and Maitre, 2007), which also encompasses biological materials in solution or deposited onto a solid substrate (Parker, 2012; Beć et al., 2020). Data is realised by recording the variation in energy as a function of the wavelength/frequency of the IR radiation as it interacts with the sample of interest (see Figure 10). Plotting the resulting signal as a function of wavenumber (or wavelength) provides a spectrum, revealing “bands” which yield chemical information as chemical bonds and functional groups have different characteristic absorption frequencies, which can then be used to identify the chemical composition of a material (Liu, 2021). The technique can therefore identify which functional groups or chemical bonds are present in a sample of interest.

Fourier-transform IR (FTIR) spectroscopy takes this one step further by illuminating a sample simultaneously with many frequencies of light and performing a Fourier transform to determine the absorption at each wavenumber (Movasaghi et al., 2008). This technique can be coupled with optical microscopy to perform hyperspectral-imaging (micro-FTIR). This generates spatially resolved FTIR maps of a sample, which can provide sub-nanometre resolution of a sample and its corresponding chemical environment (Wood et al., 1996; Wood et al., 2004; Roy et al., 2017; Vongvivut et al., 2019).

For biological applications, IR spectroscopy can interrogate biochemical signals within bio-molecules (Roggo et al., 2007; Wiercigroch et al., 2017), tissues (Rolfe, 2000; Wood et al., 2004; Movasaghi et al., 2008; Pellicer and del Carmen Bravo, 2011), single



**FIGURE 10**

Synchrotron FTIR images of human keratinocytes using macro-ATR-FTIR. (A) Image showing a cell triplet. (B) Intensity map of the integrated area of the Lipid band ( $3,010\text{--}2,837\text{ cm}^{-1}$ ). (C) Intensity map of the integrated area of the Amide I band ( $1746\text{--}1,563\text{ cm}^{-1}$ ). (B,C) Red indicates high intensity and blue indicates low intensity. Scale bars are  $20\text{ }\mu\text{m}$ .

celled organisms (Helm et al., 1991; Alvarez-Ordóñez et al., 2011; Cheeseman et al., 2021; Hartnell et al., 2021; Shaw et al., 2022) and mammalian cells (Wood et al., 1996; Arnold et al., 2003; Heraud et al., 2010; Roy et al., 2017), amongst other biological constructs (Mourant et al., 2005; Stuart, 2006; Henriques et al., 2009; Tidy et al., 2017). The spectral regions of interest are often the methylene/methyl ( $\text{CH}_2/\text{CH}_3$ ) region for lipids ( $3,000\text{--}2,800\text{ cm}^{-1}$ ), amide I and II bands for proteins ( $1705\text{--}1,600\text{ cm}^{-1}$  and  $1,567\text{--}1,448\text{ cm}^{-1}$  respectively), and fingerprint region for polysaccharides and nucleic acids ( $1,200\text{--}1,000\text{ cm}^{-1}$ ). Micro-FTIR has been recently utilised to interrogate the spatial distribution of bio-chemical properties within whole tissues (Wood et al., 2004; Heraud et al., 2010; Hackett et al., 2016), mammalian cells (Wood et al., 1996; Hackett et al., 2013; Roy et al., 2017; Vongsivut et al., 2019), and single-celled organisms (Pham et al., 2020; Cheeseman et al., 2021; Hartnell et al., 2021; Shaw et al., 2022).

For cryopreservation-based research, IR, near-IR, FTIR, and Micro-FTIR techniques can be used in several ways to evaluate the cryoprotectant—bio interactions: 1) to elucidate the bio-chemical interactions of cryoprotectant molecules with biological materials (whole cells or isolated bio-constructions) (Chopra et al., 2016); 2) to interrogate the bio-chemical alteration of cells in response to the presence of a CPA (Gläfke et al., 2012; Sieme et al., 2015; Wolkers and Oldenhof, 2016); and 3) to assess biochemical changes which occur during freeze-thaw cycles (Wolkers and Oldenhof, 2015; Giugliarelli et al., 2016; Wang et al., 2018).

While IR has the potential to interrogate many biochemical signatures at once, it is limited in absolute resolution. Data is usually an average of the interrogated area which cannot be easily controlled. For example, in the case of cells, individual components (e.g., proteins, membranes, etc.) cannot be isolated—measurements will provide an average from all contributions within a relatively large volume. Furthermore, analysis of IR data can be difficult, and may rely on minute shifts in the spectra that are difficult to quantify.

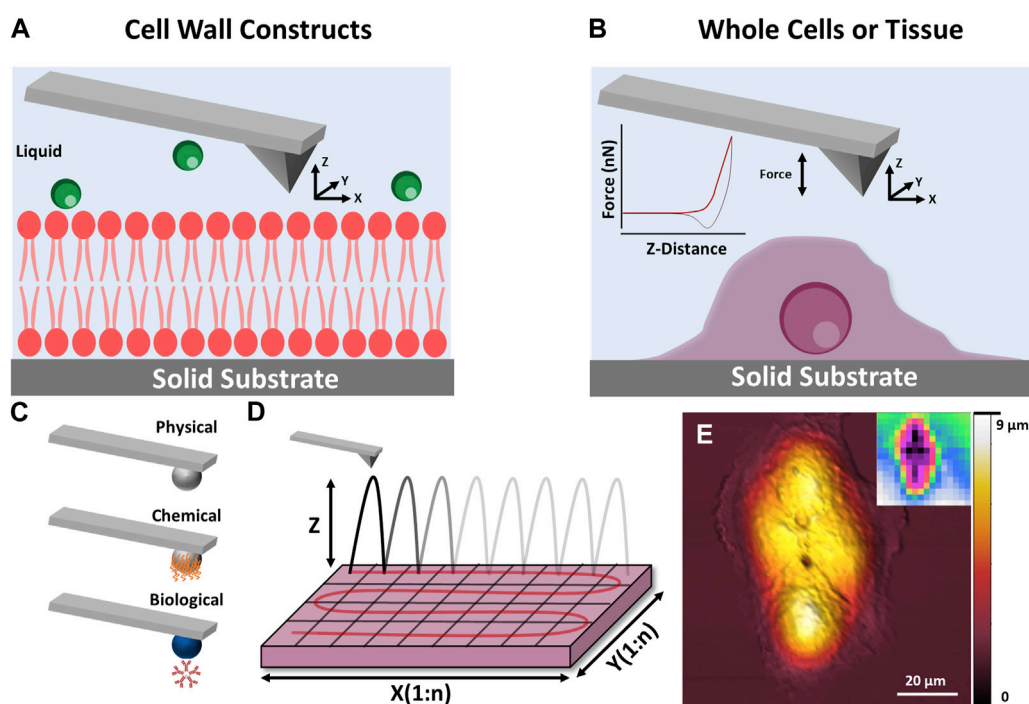
### 3.5 AFM

Atomic force microscopy (AFM) is an *in situ* scanning probe technique which can image and interrogate a system physically via a

cantilever beam with a probe attached to one end (Hansma et al., 1994; Magonov et al., 1997; Cappella and Dietler, 1999; Senden, 2001; Garcia and Perez, 2002; Butt et al., 2005; Parot et al., 2007; García, 2010). The nature of the probe is normally either a sharp-tip or a colloidal entity. Data obtained from the technique can readily provide nanometre spatial resolution (Uchihashi et al., 2005; Higgins et al., 2006; Fukuma et al., 2007; Loh and Jarvis, 2010; Ricci et al., 2014; Elbourne et al., 2015a; Hofmann et al., 2016; Kariuki et al., 2022), as well as probe nanoscale forces at a variety of interfaces (Cappella and Dietler, 1999; Butt et al., 2005; Thordarson et al., 2006; Helenius et al., 2008; Elbourne et al., 2023), including those in liquid environments.

In recent decades, the technique has progressed beyond a simple surface profiling tool, to a multi-faceted surface analysis instrument (see Figure 11), capable of probing soft matter (Wanless and Ducker, 1996; Elbourne et al., 2015a) and biological systems (Wanless and Ducker, 1996; Ducker and Wanless, 1999; Webber et al., 2001; Elbourne et al., 2013; Segura et al., 2013; Page et al., 2014; Elbourne et al., 2015b; Elbourne et al., 2016; McDonald et al., 2016), nanomechanical properties (Radmacher, 1997; Touhami et al., 2003; Parot et al., 2007; Müller and Dufrêne, 2011; Collett et al., 2019; Pham et al., 2020) and *in situ* interactions, all with nanoscale resolution (Higgins et al., 2006; Takeshi, 2010; Trewby et al., 2016; Ricci et al., 2017; Fukuma and Garcia, 2018; Trewby et al., 2019; Fukuma, 2020; Yurtsever et al., 2021). For biological systems, the AFM can probe tissues (Goksu et al., 2009; Müller and Dufrêne, 2011; Yamashita et al., 2012; Shan and Wang, 2015; Dufrêne et al., 2017), whole cells (bacteria, fungi, and mammalian) (Parot et al., 2007; Müller and Dufrêne, 2011; Dufrêne et al., 2017), bioparticles (viruses, proteins, etc.) (Collett et al., 2019; Collett et al., 2021; Boyton et al., 2022), or isolated cell constructs (membrane mimics, lipid bilayers, excised sections, etc.) (Radmacher et al., 1992; Radmacher, 1997; Goksu et al., 2009; Garvey et al., 2023).

For cryopreservation, AFM can provide qualitative and quantitative information on morphology and nanomechanical properties of whole cells, tissues, and biological extracts (natural or biomimetic). These measurements can provide *ex situ* or *in situ* information on the effect of a CPA on a given system, depending on the experimental approach. For instance, one study monitored the morphological and nanomechanical response of murine fibroblasts to varying concentrations of the traditional CPAs DMSO and glycerol before and after a freeze/thaw



**FIGURE 11**

Schematic illustration of AFM techniques which can be applied to cryopreservation research. (A,B) show the principle of AFM measurements for cell wall constructs and whole cell/tissues respectively. (C) Shows how the AFM tip can be modified to examine specific interactions including physical, chemical and biological. (D) Shows how a surface can be sequentially scanned to produce spatial maps of nanomechanical data. (E) Shows a height image of a mammalian cell taken using AFM, inset: mechanical stiffness map of the imaged cell.

cycle (Golan et al., 2018). More recently similar protocols were used to investigate the response of four human cell lines (THP-1, HaCat, PC3, and UG87-MG) to a novel CPA based on deep eutectic solvents (Bryant et al., 2022). Studies in this area are still in their infancy, however, AFM could be used to elucidate molecular mechanisms involved in cryoprotectant-membrane behaviour and structure-function properties of cryoprotectants, as well as the general effect of CPAs on whole cells and tissues.

While AFM can achieve atomic-level resolution its applications can be limited by the time required to take each measurement. As discussed above, this excludes AFM from use in examining permeability kinetics of CPAs. AFM is also limited to determining the external structure (size and shape) and does not provide information on internal structures. AFM also usually relies on samples being fixed to a surface which can be challenging for some materials. The surface can also introduce artifacts or changed interactions that would not occur in a free-floating system.

## 4 Conclusion and future research perspective

This review highlights the extensive research currently being carried out utilising soft matter techniques to overcome challenges in cryopreservation, and identify some areas where these techniques have not yet been applied but offer a lot of promise.

There is an obvious need for new CPAs (Raju et al., 2021). More than 50 years of research has demonstrated that the existing standard

CPAs (glycerol and DMSO) are unsuitable for many cell types, and are currently limiting the applications of cryopreservation. These new CPAs should be rationally designed with consideration for factors such as permeability, which can to some degree be predicted based on the “rule of 5” described in Section 2.2.2 (Lipinski et al., 1997; Yang and Hinner, 2015). However, it is not enough to test potential new CPAs using protocols that have been optimised over decades for DMSO or glycerol. Instead, new CPAs need to be fully characterised. This includes:

- Determining the ideal heating/cooling rates as well as final storage temperature to avoid freezing and ice recrystallisation (e.g., via DSC).
- Determining permeability in order to optimise the addition and removal steps of the CPAs into the cells of interest (e.g., via shrink-swell measurements).
- Determining biomolecular interactions in order to understand the mechanism of cryopreservation and of potential toxicity effects (e.g., via SANS, reflectometry, FTIR and AFM)

Utilisation of multiple techniques, such as those described in this review, will allow full exploration and characterisation of chemical, thermal, and physical properties relevant to cryopreservation protocols. This understanding will encompass length scales from just a few nanometres up to several microns. Ultimately these explorations will lead to the development of structure-property relationships for CPAs and improved cryopreservation protocols.

Characterisation and optimisation will allow the proper utilisation of new CPAs and may allow the replacement of the current gold

standards of DMSO and glycerol for some applications. This in turn will have unprecedented benefits in numerous fields. First, application of new CPAs may allow the storage of new cell types, which can be used for biomedical therapies as well as scientific research. Second, novel CPAs may facilitate the storage of more complex materials such as tissues, plant samples and maybe even organs which would have unprecedented benefits for the medical sector.

This review has focused on some of the less widely used techniques which can help address the challenges in cryopreservation. There are of course many other useful techniques, including the standard techniques detailed in Table 2, as well as well-known chemical techniques such as electron paramagnetic resonance and nuclear magnetic resonance, but these are beyond the scope of this review. The techniques reviewed here have the potential to provide a detailed understanding of the underlying mechanisms of cryopreservation which can then be used for the rational design of cryopreservation protocols. Armed with these techniques it is likely that we will see rapid advancement of the cryopreservation field in the near future.

## Author contributions

Writing—original draft preparation, SB; writing—review and editing, SB, AE, TG, and GB; All authors contributed to the article and approved the submitted version.

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## Conflict of interest

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